

ENTEROPATHOGENICITY OF *Aeromonas hydrophila* AND
Plesiomonas shigelloides: PREVALENCE AMONG
INDIVIDUALS WITH AND WITHOUT DIARRHEA
IN THAILAND

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OBJECTIVES : To evaluate the enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides* in Thailand.

BACKGROUND : Although the role of *Aeromonas hydrophila* and *Plesiomonas shigelloides* as enteric pathogens has not been conclusively proven, diarrheal disease thought to be caused by *A. hydrophila* (15, 20, 24) and *P. shigelloides* have been described (23). Some workers believe that these organisms are not even potential human pathogens, but resident aquatic microorganisms which occasionally colonize man. Other investigators suspect that the isolation of aeromonads from feces of patients with diarrhea is evidence that some strains at least are primary intestinal pathogens (17-21).

In the United States Von Graevanitz and Mench (24) reported two cases of diarrhea associated with *Aeromonas* isolates in the stool, but also recovered these organisms from the stools of five asymptomatic individuals. In recent years numerous authors have published reports of acute, occasionally severe diarrheal syndromes associated with the recovery of *Aeromonas* species in the stool (20, 23, 24). These illnesses were usually self-limited and in most cases other possible etiologies were excluded. A recently reported case of a patient with prolonged diarrhea associated with repeated isolation of *A. hydrophila* from feces in the absence of other enteric pathogens with recovery after appropriate antimicrobial therapy, provides further evidence to support the enteropathogenicity of this organism (15). On the Indian subcontinent *Aeromonas* species have been implicated in episodes of cholera-like disease (3, 16) and *P. shigelloides* have been implicated in two large epidemics of diarrheal disease in Japan (23). Although the rate of asymptomatic bowel carriage has been reported to be very low (2, 10, 17), there have been few

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attempts to compare the prevalence of aeromonads in patients with diarrhea with the prevalence in controls without gastrointestinal symptoms.

To determine the enteropathogenicity of *A. hydrophila* and *P. shigelloides*, we compared the prevalence of these organisms in different populations of Thais and Americans in Thailand. Isolates from patients with and without diarrhea were examined with assays previously used to evaluate enteropathogenicity of other enteric bacteria.

METHODS Stools from patients with diarrhea and controls were collected from a hospital in Bangkok, Thailand.

Source of isolates: Three Thai populations and two groups of American Peace Corps volunteers who had recently arrived in Thailand were studied. The characteristics of these populations are given in Table 1. (P. Echeverria, N.R. Blacklow, L.B. Sanford, and G.G. Cukor. A study of travelers' diarrhea among American Peace Corps volunteers in rural Thailand. J. Infect. Dis., in press). Among Thais an episode of diarrhea was defined as twice the number of normal bowel movements accompanied with stools assuming the shape of the container occurring for less than 48 hours. In studies of diarrhea among American Peace Corps volunteers three or more watery stools or two watery stools accompanied by either nausea, vomiting, abdominal cramping, fever, or prostration within a 24 hour period was defined as an episode of travelers' diarrhea. Formed stools were collected from asymptomatic volunteers in the first group on arrival and after they had been in Thailand five weeks. Similar specimens were collected from the second group on arrival and after they have been in Thailand two and five weeks.

Bacteriology: Stool specimens were cultured directly on MacConkey and Heckteon media (Difco, Detroit, MI) at 37°C for 24 hours. Colonies that did not ferment lactose on MacConkey media, flat green (*P. shigelloides*), convex green or yellow-orange colonies (*A. hydrophila*) on Heckteon media, or colonies which did not ferment lactose on desoxycholate media were identified by methods summarized in Table 2 (12). In addition, any of ten lactose positive colonies, selected from the MacConkey media, which were indole negative, metabolized citrate, and were methyl red negative were further identified. Colonies which initially appeared to be *A. hydrophila* were inoculated into alkaline peptone water with 7% sodium chloride to distinguish *A. hydrophila* from *Vibrio fluvalis* (group "F" or EF6 vibrios) (10).

In vitro test: Twofold dilutions in PBS, pH=7.4 of sterile filtrates of aeromonads grown in trypticase soy broth (BBL, Cockeysville, MD) with 0.6% yeast extract (Difco) at 37°C on a roller drum (eight revolutions per minute) for 18 hours were tested for hemolysis of sheep and rabbit erythrocytes and cytotoxicity in Y-1 adrenal cell tissue cultures (4). *A. hydrophila* and *P. shigelloides* were tested for mannose resistant hemagglutination of human (group A) and bovine erythrocytes (9).

P. shigelloides were tested for agglutination in *Shigella* grouping antisera (Difco, Detroit, MI). Isolates that agglutinated in *Shigella* antisera and cytotoxic *A. hydrophila* were tested in the Sereny test (20).

In vivo tests : Whole cultures (10^9 /ml) of *A. hydrophila* or *P. shigelloides* were tested for their ability to distend ligated rabbit ileal loops after seven and 18 hours (15), and three to four day old suckling mouse intestine after four hours (6). Tissue sections of suckling mouse and rabbit ileal loops were fixed in ten percent formalin, Giemsa stained, and examined microscopically.

9 After 24 hours starvation, groups of five adult rhesus monkeys were fed 10^9 *P. shigelloides* isolated from a patient with a severe diarrhea, or 10^9 cytotoxic *A. hydrophila* from another patient with watery stools and abdominal cramps. The inocula were administered by nasogastric tube 20 minutes after each animal received three grams of NaHCO_3 in 20 mls of distilled water. Monkeys were observed four times a day for five days and rectal temperature were measured twice daily. Another group of five monkeys were pretreated for five days with 40 mg of trimethoprim and 200 mg of sulfamethoxazole twice daily, starved for 24 hours, and fed 10^9 cytotoxic *A. hydrophila* 20 minutes after receiving sodium bicarbonate.

RESULTS :

Prevalence of Aeromonas : *A. hydrophila* and *P. shigelloides* were uncommon in Thai infants cultured within the first week of life and the isolation rate among asymptomatic inhabitants of Soongnern increased with age. The percentage of Thais with and without diarrhea from whom *A. hydrophila* and *P. shigelloides* were isolated was not significantly different (Table 3). In the first group of 35 Peace Corps volunteers, *A. hydrophila* was isolated significantly more frequently from individuals during episodes of travelers' diarrhea than when volunteers were well after five weeks (12/39 vs 3/35) ($p < 0.025$). One volunteer had two separate episodes of travelers' diarrhea associated with *A. hydrophila*. This pathogen was isolated from two individuals who were not infected with viral, bacterial, or parasitic pathogens within 24 hours of the onset of travelers' diarrhea. *P. shigelloides* were not statistically associated with episodes of travelers' diarrhea more often than when volunteers were asymptomatic after five weeks in Thailand (13/39 vs 6/35).

In the second group of 33 volunteers *A. hydrophila* was isolated during episodes of travelers' diarrhea more frequently than when individuals were well and submitted formed stools after two and five weeks in Thailand (8/14 vs 9/59) ($p < 0.001$). In seven episodes of travelers' diarrhea *A. hydrophila* was the only possible enteric pathogen found. Again *P. shigelloides* was not isolated during episodes of travelers' diarrhea more often than when volunteers were well (6/14 vs 15/59). Tenesmus and anorexia were the most common symptoms of nine volunteers with travelers' diarrhea from whom *A. hydrophila* was isolated as the sole potential enteric pathogen (Table 4).

Biochemical characteristics : The biochemical characteristics of *A. hydrophila* isolated from patients with diarrhea and others without gastrointestinal symptoms were similar. There was, however, a correlation between cytotoxicity and lysine decarboxylase, the Voges-Proskauer reaction, and metabolism of citrate in the API 20E system.

Assays for enterotoxigenicity : Seventy-one percent (30/42) of culture supernatants of *A. hydrophila* isolated from individuals with diarrhea and 64 percent (18/28) isolated from those who were passing formed stools were cytotoxic to Y-1 adrenal cells and hemolyzed rabbit erythrocytes. Whole cultures of thirty-eight percent (16/42) of strains isolated from individuals with diarrhea and 29 percent (8/28) from those without gastrointestinal complaints distended suckling mouse intestine (gut to remaining body weight ratio >0.083). Sterile supernatants of 13 of 24 whole cultures of *A. hydrophila* which distended suckling mouse intestine were also positive in the mouse assay. Distension of mouse intestine did not occur when cultures were heated at 100°C for ten minutes. The results of these assays are summarized in Table 5.

None of 44 *A. hydrophila* or eleven *P. shigelloides* tested caused mannose resistant agglutination of human or bovine erythrocytes. Histologic examination of mouse intestine infected with six cytotoxic *A. hydrophila* did not reveal bacteria adhering to small intestinal brush borders. Cytotoxic strains of *A. hydrophila* caused blunting of villi and degeneration of enterocytes, but no intracellular organisms were seen in mouse intestine. Ten cytotoxic *A. hydrophila* strains were also negative in the Sereny test (20).

Eight of nine cytotoxic and none of five non-cytotoxic *A. hydrophila* caused distension in ligated rabbit ileal loops after 18 hours. Two of the cytotoxic strains which distended rabbit intestinal loops after 18 hours also caused distension after seven hours. Exposure of rabbit intestine to eight cytotoxic *A. hydrophila* caused epithelial damage of the villi associated with a polymorphonuclear infiltrate extending through the muscularis mucosa (Fig. 1). Bacteria were found in the subepithelial space under intact mucosa (Fig. 2). The one cytotoxic *A. hydrophila* which did not distend rabbit intestine also did not cause epithelial damage of the mucosa.

Monkeys fed 10^9 cytotoxic *A. hydrophila* following 24 hours starvation and oral administration of sodium bicarbonate failed to develop diarrhea over the ensuing five days. To decrease any interference by the normal aerobic stool flora another five monkeys were treated with trimethoprim and sulfamethoxazole twice daily for five days and then infected with 10^9 cytotoxic *A. hydrophila*. None of these monkeys developed diarrhea.

None of 27 *P. shigelloides* tested were cytotoxic to Y-1 adrenal cell tissue cultures, hemolyzed rabbit or bovine erythrocytes, distended suckling mouse intestine after four hours, or distended ligated rabbit intestinal loops after 18 hours. An isolate from an individual with severe diarrhea did not cause diarrhea in five rhesus monkeys. Twenty-six percent (7/27) of *P. shigelloides* isolates did agglutinate in either group A and D (2), C (1), or C and D (4) *Shigella* grouping antisera, however none of the cross-agglutinating isolates were positive in the Sereny test (20).

This study is complete.

Table 1. Characteristics of Populations Cultured in Thailand

<u>Individuals with diarrhea</u>	<u>Description</u>	<u>Individuals without diarrhea</u>	<u>Description</u>
<u>Bangkok</u>			
<u>Children's Hospital</u>			
105 children	children 0-2 years of age admitted to the rehydration ward May-July 1979	74 convalescent children	same children cultured 3-4 weeks after their episode of diarrhea
		100 children	children 0-2 years of age without diarrhea for two weeks seen at the outpatient department May-July 1979
<u>Phra Mongkutklao Hospital</u>			
18 newborns	newborns less than one week of age with diarrhea May-July 1979	66 newborns	newborns cultured within 7 days after delivery May-July 1979
<u>Soongnern</u>			
<u>Soongnern Hospital</u>			
30 children 0-2 years of age	children admitted to the hospital for rehydration May-July 1980	101 children 0-2 years of age	children without diarrhea for the previous two weeks living in villages in Soongnern May-July 1980
7 children 2-10 years of age	"	51 children 2-10 years of age	"
9 children 10-20 years of age	"	-	none cultured
56 adults (>20 years of age)	adults admitted to the hospital for rehydration May-July 1980	51 adults (>20 years of age)	adults without diarrhea for the previous two weeks living in villages in Soongnern May-July 1980
<u>American Peace Corps volunteers</u>			
Group I			
39	episodes of travelers' diarrhea among 35 volunteers during their first five weeks in Thailand	35	volunteers after five weeks in Thailand
Group II			
14	episodes of travelers' diarrhea among 33 volunteers during their first five weeks in Thailand	26	volunteers after two weeks in Thailand
		33	volunteers after five weeks in Thailand

Table 2. Identification of *Aeromonas hydrophila* and *Plesiomonas shigelloides*.

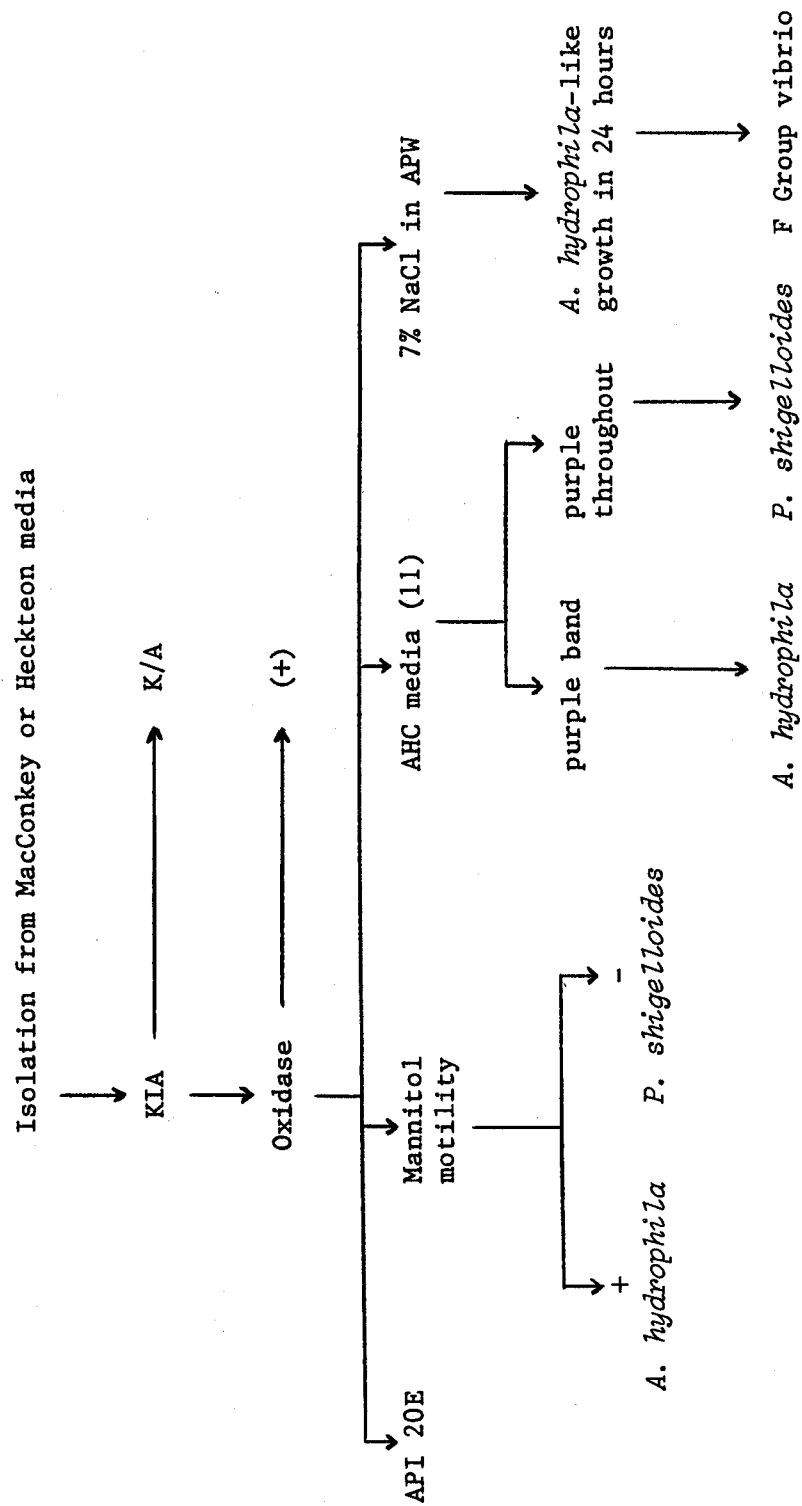


Table 3. Prevalence of *Aeromonas hydrophila* and *Plesiomonas shigelloides* in populations in Thailand.

<u>Indigenous population</u>	<u><i>Aeromonas hydrophila</i> Diarrhea</u>	<u>Controls</u>	<u>Signifi- cance</u>	<u><i>Plesiomonas shigelloides</i> Diarrhea</u>	<u>Controls</u>	<u>Signifi- cance</u>
<u>Children's Hospital</u>						
0-2 years	9/105 (9%)	6/74* (9%)	NS	4/105 (4%)	3/74 (4%)	NS
		8/90 (8%)	NS		2/90 (2%)	NS
<u>Soongnern District</u>						
0-2 years	6/30 (20%)	8/101 (8%)	NS	4/30 (13%)	3/101 (3%)	NS
2-10 years	1/7 (14%)	8/51 (16%)	NS	3/7 (43%)	5/51 (10%)	NS
10-20 years	2/9 (22%)	NC	NT	3/9 (33%)	NC	NT
Adults	19/56 (34%)	14 (51 (27%)	NS	20/56 (38%)	12/51 (24%)	NS
<u>Phramongkutkloao Hospital</u>						
Newborns	0/18 (0%)	1/84 (1%)	NS	0/18	0/84	NS

* 74 of the 105 children with diarrhea cultured 3-4 weeks after their acute episodes.

Table 4. Summary of travelers' diarrhea in nine volunteers from whom *A. hydrophila* was found as the only potential enteric pathogen.

<u>Characteristics of patients with travelers' diarrhea</u>	<u>No. of volunteers</u>
<u>Stools/day</u>	
< 2	3
3 - 5	4
6 - 10	2
<u>Symptoms</u>	
Headache	3
Photophobia	1
Anorexia	7
Malaise	6
Chills	2
Fever	3
Temp. >37.8°C	1
Abdominal cramps	7
Nausea	3
Vomiting	1
Prostration	2
Flatulence	3
<u>Duration of diarrhea</u>	
1 - 2 days	3
3 - 4 days	2
5 - 6 days	2
> 7 days	2

Table 5. *In vitro* tests with *Aeromonas hydrophila*.

<u>Test</u>	<u>Diarrhea (42)*</u>	<u>No diarrhea (28)</u>
Cytotoxin	30 ⁺ /42(71%)	18/28(54%)
Hemolysis of rabbit RBC	30 ⁺ /42(71%)	18/28(54%)
<u>Distension of mouse intestine</u>		
Whole culture	16 ^o /42(38%)	8/28(29%)
Culture filtrate	9 ^o /42(21%)	4/28(14%)

* Number of isolates (one isolate/patient/episode of diarrhea).

+ All isolates which were cytotoxic to Y-1 adrenal cells hemolyzed rabbit RBC.

^o All isolates which distended suckling mouse intestine were cytotoxic to Y-1 adrenal cells.

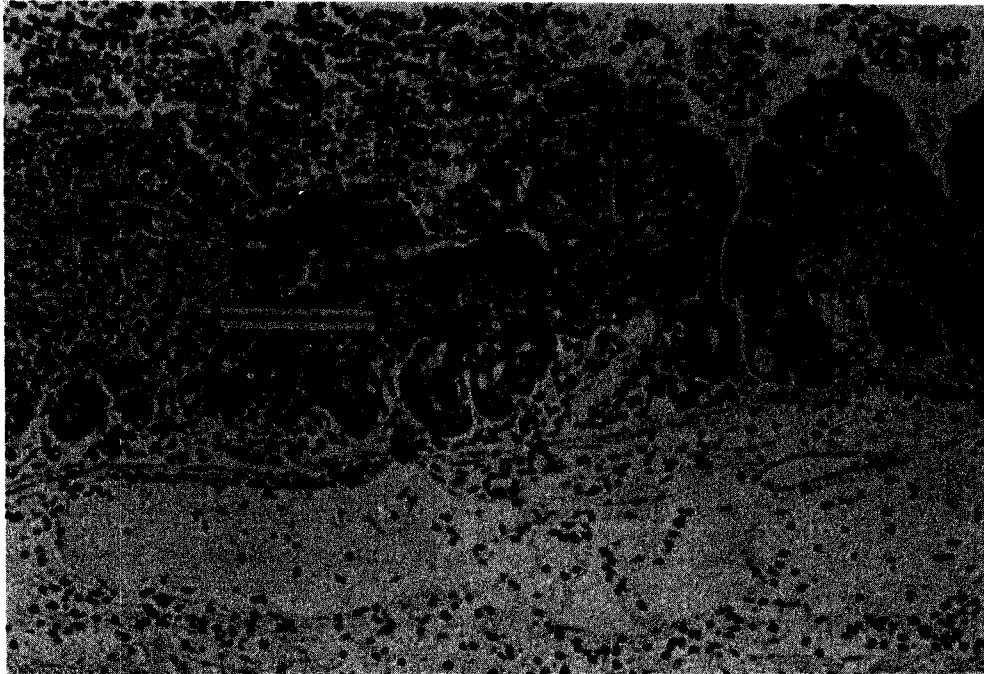


Figure 1. Tissue section of a rabbit ileal loop seven hours after challenge with a cytotoxic *A. hydrophila* (6Y). The villus on the right is broad and blunt, has no mucus-containing cells, and has a hypercellular lamina propria. The cellular infiltrate consisting largely of polymorphonuclear cells extends through muscularis mucosa and into a widened edematous submucosa in which there are dilated lymphatics and numerous polymorphonuclear cells. Epithelial damage of the villi on the left is extensive with an overlay of this portion by a hemorrhagic and leucocytic exudate. Damage extends to and into the crypt level of the mucosa. There is margination of the submucosal lymphatics by polymorphonuclear cells.
(Giemsa stain) Bar equals 100 μ m

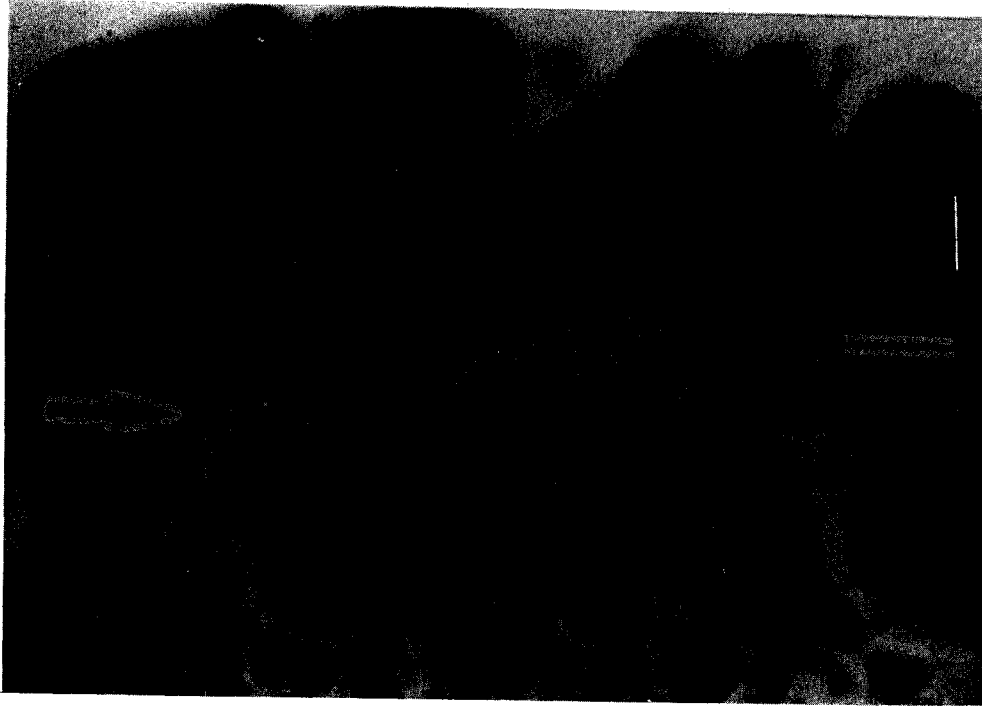


Figure 2. Tissue section of a rabbit ileum seven hours after infection with cytotoxic *A. hydrophila* (6Y). The epithelium is intact and consists of cuboid and columnar cells. There is a cellular infiltration of the epithelium and lamina propria, predominantly mononuclear, with subepithelial edema and bacteria (see arrow) in the subepithelial space. (Giemsa stain) Bar equals 20 μ m

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